JOURNAL OF VIROLOGY, Dec. 1991, p. 6597–6603
0022-538X/91/126597-07$02.00/0

Vol. 65, No. 12

N-Terminal Truncation of the Scrapie-Associated Form of PrP by Lysosomal Protease(s): Implications Regarding the Site of Conversion of PrP to the Protease-Resistant State

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Received 24 June 1991/Accepted 28 August 1991

Scrapie and related transmissible spongiform encephalopathies result in the accumulation of a protease-resistant form of an endogenous brain protein called PrP. As an approach to understanding the scrapie-associated modification of PrP, we have studied the processing and sedimentation properties of protease-resistant PrP (PrP-res) in scrapie-infected mouse neuroblastoma cells. Like brain-derived PrP-res, the neuroblastoma cell PrP-res aggregated in detergent lysates, providing evidence that the tendency to aggregate is an intrinsic property of PrP-res and not merely a secondary consequence of degenerative brain pathology. The PrP-res species had lower apparent molecular masses than the normal, protease-sensitive PrP species and were not affected by moderate treatments with proteinase K. This suggested that the PrP-res species were partially proteolyzed by the neuroblastoma cells. Immunoblot analysis of PrP-res with a panel of monospecific anti-PrP peptide sera confirmed that the PrP-res species were quantitatively truncated at the N terminus. The metabolic labeling of PrP-res in serum-free medium did not prevent the proteolysis of PrP-res, showing that the protease(s) involved was cellular rather than serum-derived. The PrP-res truncation was inhibited in intact cells by leupeptin and NH_4Cl. This provided evidence that a lysosomal protease(s) was involved, and therefore, that PrP-res was translocated to lysosomes. When considered with other studies, these results imply that the conversion of PrP to the protease-resistant state occurs in the plasma membrane or along an endocytic pathway before PrP-res is exposed to endosomal and lysosomal proteases.

The transmissible spongiform encephalopathies form a group of neurodegenerative diseases which include scrapie and bovine spongiform encephalopathy in animals and Creutzfeldt-Jakob disease, kuru, and Gerstmann-Straussler syndrome in humans. A distinguishing feature of these diseases is the accumulation in the brain of a disease-specific, protease-resistant form of an endogenous protein, PrP (6, 10, 18, 23, 36). Unlike the normal, protease-sensitive PrP (PrP-sen), the protease-resistant PrP (PrP-res) can aggregate into amyloidlike fibrils (19, 21, 23, 37, 43, 56) and is a major component of brain fractions enriched for scrapie infectivity (6, 23, 25). Since the infectious agents of the transmissible spongiform encephalopathies are resistant to treatments harmful to nucleic acids, it has been postulated that these agents are devoid of nucleic acid and composed primarily of protein (1, 26, 41, 42). More recently, PrP-res, or the fibril it forms, was proposed to be the transmissible agent of these diseases (6, 23, 36, 38) or a product of a virus-induced amyloidosis (9, 20). However, it is not clear whether PrP-res is the transmissible agent itself, a component of the agent, or a byproduct of the infection which happens to cofractionate with scrapie infectivity.

A number of studies have suggested that the differences between PrP-res and PrP-sen arise at the posttranslational level (2, 8, 15, 18, 40). Strong evidence for this has been obtained recently (16). Several posttranslational modifications of PrP, including the addition of N-linked glycans and a glycophosphatidylinositol moiety, are known to occur (7, 14, 15, 35, 50, 51). Although some investigators have stated that covalent differences between the PrP isoforms must exist (22), no scrapie-specific covalent modifications of PrP have been identified. Conformational studies have indicated that PrP-res fibrils resemble other amyloids in being predominantly β-sheet (17), but it is not known whether this conformation differs from that of PrP-sen. Thus, the molecular basis of the difference between PrP-res and PrP-sen remains unknown.

Scrapie-infected (sc^+) murine neuroblastoma cells have become a prototypic in vitro system for investigating PrP biosynthesis and scrapie (12, 44–46). Studies of PrP biosynthesis in both sc^+ and uninfected (sc^-) neuroblastoma cells have shown that PrP-sen is located on the cell surface in multiple forms that differ in the amount of N-linked glycan they contain (14). All of these forms are linked to the cell surface by a phosphatidylinositol moiety and can be released from intact cells with phosphatidylinositol-specific phospholipase C (PIPLC) (14, 51). PrP-res, which is found in sc^+ clones only (11, 13), is derived from a protease- and PIPLC-sensitive cell surface precursor that, so far, is indistinguishable from PrP-sen (16). However, mature PrP-res accumulates intracellularly and is resistant to release from intact cells by PIPLC or proteases (8, 13, 53). In the present study, we have further analyzed the metabolic fate of PrP-res. Our results show that PrP-res aggregates and is quantitatively truncated at the N terminus by lysosomal, and possibly endosomal, proteases within these cells. These findings help to define the subcellular site of the formation of PrP-res and provide evidence that PrP-res is translocated to lysosomes.

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MATERIALS AND METHODS

Neuroblastoma cells. The sc^+ and sc^- mouse neuroblastoma clones were established and maintained as described previously (45, 46).

Antibodies. Synthetic peptides corresponding to amino acid residues 23 to 37, 89 to 103, and 218 to 232 of the full mouse PrP sequence with additional cysteine residues at the N termini were kindly made by Michael Buchmeier (peptide 89-103; Scripps Clinic and Research Foundation, La Jolla, Calif.) and John Coligan (peptides 23-37 and 218-232; National Institute of Allergy and Infectious Diseases, Bethesda, Md.). The peptides were coupled via the cysteine sulfhydryl group to keyhole limpet hemocyanin and inoculated into rabbits as previously described (56).

Metabolic labeling. Flasks (25 cm^2) of cells between 50 and 90% confluent were preincubated for 60 min in 2 ml of methionine-free minimum essential medium (GIBCO, Grand Island, N.Y.) containing 1% dialyzed fetal bovine serum (FBS) except in the experiment (see Fig. 4) in which the FBS was purposefully omitted from this and subsequent incubation media. Then, 350 to 500 μCi of [^{35}S]methionine (Dupont-NEN) or Tran^{35}S-label (ICN) was added to each flask for the designated times in the presence or absence of lysosomal protease inhibitors (as described in the figure legends). The cells were washed three times with phosphate-buffered balanced salts solution, chase-incubated in complete minimum essential medium with 10% FBS, and washed twice with ice-cold phosphate-buffered balanced salts solution. The cells were then lysed in a solution of ice-cold 0.5% Triton X-100, 0.5% sodium deoxycholate 5 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.7 μg of pepstatin per ml, and 0.5 μg of leupeptin per ml (1 ml per flask). Phenylmethylsulfonyl fluoride (PMSF; 0.5 mM) was also included in the lysing buffer when the lysates were not subsequently treated with proteinase K. Nuclei and debris were removed by centrifuging at 1,000 × *g* for 5 min (4°C). The postnuclear supernatants were treated as described in the figure legends and ultimately either centrifuged at 230,000 × g_{max} for at least 45 min to pellet aggregates or precipitated by the addition of 4 volumes of cold methanol, cooled at −20°C for at least 30 min, and then collected by centrifugation at 13,000 × *g*. In the experiment shown in Fig. 5, the 230,000 × *g* pellets were washed once with a solution containing 10% Sarkosyl (*N*-laurylsarcosinate), 10 mM Tris-HCl, 1 mM EDTA, 133 mM NaCl, and 1 mM dithiothreitol (pH 8.3) to reduce nonspecific background in the subsequent immunoprecipitations.

Immunoprecipitation. The pellets resulting from the metabolic labeling experiments were resuspended by sonication in detergent-lipid-protein complex (DLPC) buffer (8) supplemented with 0.5 mM PMSF, 0.7 μg of pepstatin per ml, and 0.5 μg of leupeptin per ml. An anti-PrP peptide 89-103 serum was diluted 1:250 to 1:500 into the PrP-DLPC solutions and incubated at 4°C overnight. Immune complexes were collected by binding to protein A-Sepharose (30 μl of 10% [wt/vol] protein A-Sepharose per 2 μl of undiluted antiserum in sample) for 30 to 60 min at 4°C. The beads were then washed as previously described (8), except that the wash times were reduced to 1 to 3 min. Bound proteins were released by boiling the beads in 30 μl of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 5% SDS and 4% β-mercaptoethanol (33).

SDS-PAGE, fluorography, and immunoblotting. Proteins were separated by SDS-PAGE (12.5% acrylamide) as previously described (33). Gels of radiolabeled samples were processed for fluorography as previously described (13). For analyses by immunoblotting, proteins were electroblotted onto Immobilon-P (Millipore, Bedford, Mass.) by using a standard buffer (54) supplemented with 0.01% SDS. The Immobilon-P filter was blocked with 5% nonfat dried milk in 10 mM Tris-HCl (pH 8.0)–150 mM NaCl–0.05% Tween 20 (TBST). The filter was incubated for 2 h at ambient temperature with the appropriate rabbit antiserum diluted 1:1,000 to 1:2,000 in TBST. After washing in TBST, the filter was stained with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Protoblot; Promega, Madison, Wis.).

Partial purification of PrP-res. PrP-res was partially purified by a modified version of a procedure described previously for the extraction of full-length PrP-res (except for the signal peptide) from sc^+ brain tissue (29). Confluent 150-cm^2 flasks of sc^+ neuroblastoma clones were rinsed twice in phosphate-buffered saline, lysed in a solution containing 7.5 ml of ice-cold 10% *N*-laurylsarkosinate per flask, 1 mM PMSF, 0.7 μg of pepstatin per ml, 5 μg of leupeptin per ml, and 10 mM sodium phosphate (pH 7.6), and homogenized with several 10-s bursts of a high-speed tissue homogenizer (Tekmar, Cincinnati, Ohio). Although the protease inhibitors were not all identical to those used in a previous study (29), we have shown by N-terminal sequencing that our inhibitors are effective in this procedure at preventing the N-terminal truncation of brain tissue-derived PrP-res (data not shown). The homogenate was centrifuged at 22,000 × *g* for 30 min at 10°C. The supernatant was then centrifuged at 215,000 × *g* for 150 min at 10°C. The pellet was frozen at −20°C, and then aliquots were boiled in SDS-PAGE sample buffer prior to immunoblot analyses.

RESULTS

Aggregation of PrP-res. To compare the aggregation properties of PrP-res and PrP-sen derived from sc^+ tissue culture cells, detergent lysates of pulse-labeled sc^+ neuroblastoma cells were ultracentrifuged and the resulting pellets and supernatants were analyzed for immunoprecipitable PrP species that were either sensitive or resistant to proteinase K (Fig. 1). The cells were chased for 2 and 24 h to allow optimal labeling of PrP-sen and PrP-res, respectively (8, 14, 16). The PrP species in the 230,000 × *g* supernatants were proteinase K sensitive and, thus, were PrP-sen by definition (Fig. 1A). After longer fluorographic exposure of the gel (Fig. 1B), PrP species were also observed in the 230,000 × *g* pellets. These species were resistant to proteinase K and, thus, were PrP-res. This result indicated that the detectable PrP-res was aggregated in the detergent lysates and could be separated quantitatively from the detergent-soluble PrP-sen by centrifugation.

Comparison of the relative amounts of label maximally incorporated into PrP-res and PrP-sen by densitometric scanning of the fluorograms in Fig. 1 indicated that after the 24-h chase, the label in PrP-res was only approximately 3% of the label initially found in PrP-sen with the 2-h chase. Therefore, only a small proportion of the total PrP synthesized entered the PrP-res pool.

Proteolytic processing of PrP-res. Although the N terminus of most of the PrP-res isolated from sc^+ brain tissue begins at the signal peptidase cleavage site, further truncations at the N terminus can occur in vivo or upon treatment with moderate concentrations of proteases in vitro (5, 29, 30, 55). For instance, proteinase K treatment removes 58 amino acid residues from the N terminus of mouse brain cell-derived

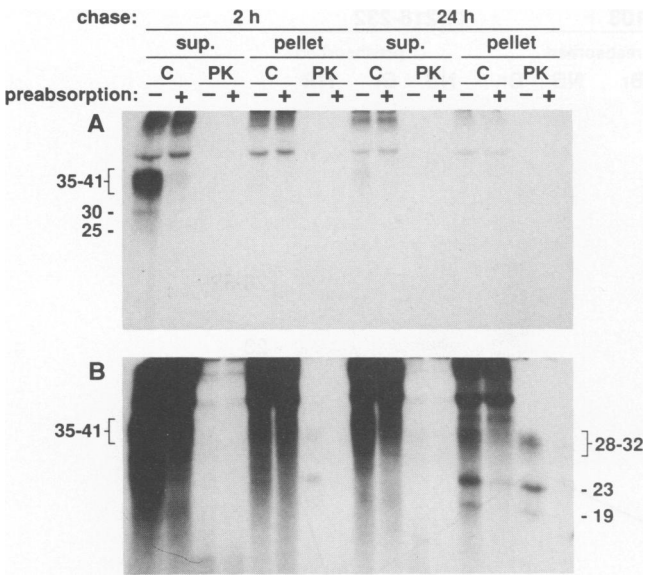

FIG. 1. Metabolic labeling and aggregation state of PrP-res. Two flasks of sc^+ neuroblastoma cells were labeled for 2.5 h with L-[^{35}S]methionine, chased with complete growth medium for 2 or 24 h, and lysed as described in Materials and Methods. Half of the postnuclear supernatants from the lysates were treated with 20 μg of proteinase K (PK) per ml for 30 min at 37°C, and the control half (C) was left on ice. The postnuclear supernatants were then centrifuged at 230,000 × *g* for 45 min at 4°C. The 230,000 × *g* supernatants (sup.) were methanol precipitated. The 230,000 × *g* pellets were washed once with ice-cold water. The methanol precipitates and the 230,000 × *g* pellets were resuspended in DLPC buffer and immunoprecipitated as described in Materials and Methods. Bands containing the specific PrP peptide 89-103 epitope were identified as those that were not immunoprecipitated when the anti-PrP peptide antiserum (R9) was preabsorbed with the appropriate synthetic PrP peptide (50 ng/μl of serum) that was used to make the antigen. Panels A and B represent 1-day and 20-day fluorographic exposures of the gel, respectively. The sizes (in kilodaltons) of the PrP bands were estimated by comparison to molecular mass standards and are indicated on the left and right sides of the figure for PrP-sen and PrP-res bands, respectively.

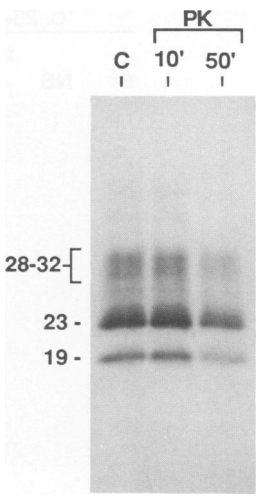

FIG. 2. Effects of proteinase K (PK) treatment on neuroblastoma cell PrP-res. Partially purified PrP-res was isolated from sc^+ neuroblastoma cells under conditions that have been shown to be effective in isolating full-length PrP-res from sc^+ brain tissue. Aliquots containing less than 1 μg of protein were suspended in 0.5% Sarkosyl–20 mM Tris-HCl, pH 7.7, and treated with proteinase K (2 μg/ml) for the designated period of time and immunoblotted with the anti-PrP peptide 89-103 serum, as described in Materials and Methods. The positions of the PrP-res species are designated in kilodaltons. C, control.

PrP-res and decreases the apparent molecular masses of the PrP-res species by approximately 6 kDa (30). However, analysis of the sc^+ neuroblastoma cell PrP-res in 230,000 × *g* pellets as shown in Fig. 1B indicated that the proteinase K treatment eliminated background proteins but did not appear to alter the SDS-PAGE mobilities or relative intensities of the individual PrP-res bands. This, and the fact that the PrP-res species, as a group, had lower apparent molecular masses than the PrP-sen species, provided evidence that the PrP-res species were already truncated at the N terminus within the neuroblastoma cells.

Since background proteins partially obscured the detection of the 28- to 32-kDa PrP-res and perhaps larger PrP-res species in crude 230,000 × *g* pellets (Fig. 1B), we also analyzed the proteinase K effect on PrP-res partially purified by a method shown to be effective for isolating full-length PrP-res from sc^+ brain tissue (29). This experiment confirmed that proteinase K had no effect on the mobility or relative intensity of the PrP-res bands (Fig. 2). The activity of the proteinase K was confirmed by the fact that, with prolonged treatment (50 min), the PrP-res staining was decreased as we have documented previously (39). Presumably the slow digestion of the epitope (residues 89 to 103), which corresponds to the relatively proteinase K-resistant region of brain tissue-derived PrP-res molecules (30), accounts for this observation.

Immunoblot analysis of PrP-res with monospecific antisera. To verify that the neuroblastoma PrP-res species were truncated at the N terminus, we analyzed PrP-res by immunoblot with monospecific antisera to synthetic peptides corresponding to the N terminus and two other sequences of the PrP polypeptide. Figure 3 shows that all three of the major PrP-res bands bound antibodies against peptides 89-103 and 218-232 which bracket the sequence of the proteinase K-resistant core of brain cell-derived PrP-res (30). However, none of the neuroblastoma PrP-res bands reacted with the antiserum against the N-terminal peptide starting at the signal peptidase cleavage site (residues 23 to 37). The activity of the anti-peptide 23-37 was demonstrated by the labeling of a subset of bands from a partially proteinase K-digested preparation of mouse brain PrP-res which contained both full-length and truncated forms of PrP-res. These results indicated that the PrP-res from the sc^+ neuroblastoma cells was quantitatively truncated at the N terminus. The PrP-res species were 6 to 7 kDa smaller than the corresponding PrP-sen species (Fig. 1A and B), suggesting that PrP-res lacked approximately the same number of amino acid residues, i.e., 58, as proteinase K-treated PrP-res from sc^+ mouse brain.

Cellular versus serum origin of proteolytic activity. Since at least the precursor of PrP-res resides on the cell surface (16), it was possible that serum proteases in the growth medium, rather than cellular proteases, were responsible for the proteolysis of PrP-res. To analyze this, we labeled PrP-res in serum-free medium. This had no effect on the SDS-PAGE migration of the PrP-res species observed, which comigrated with proteinase K-treated PrP-res bands (Fig. 4). Thus, the protease(s) responsible for truncating PrP-res is not derived from the FBS and, by default, must be cellular.

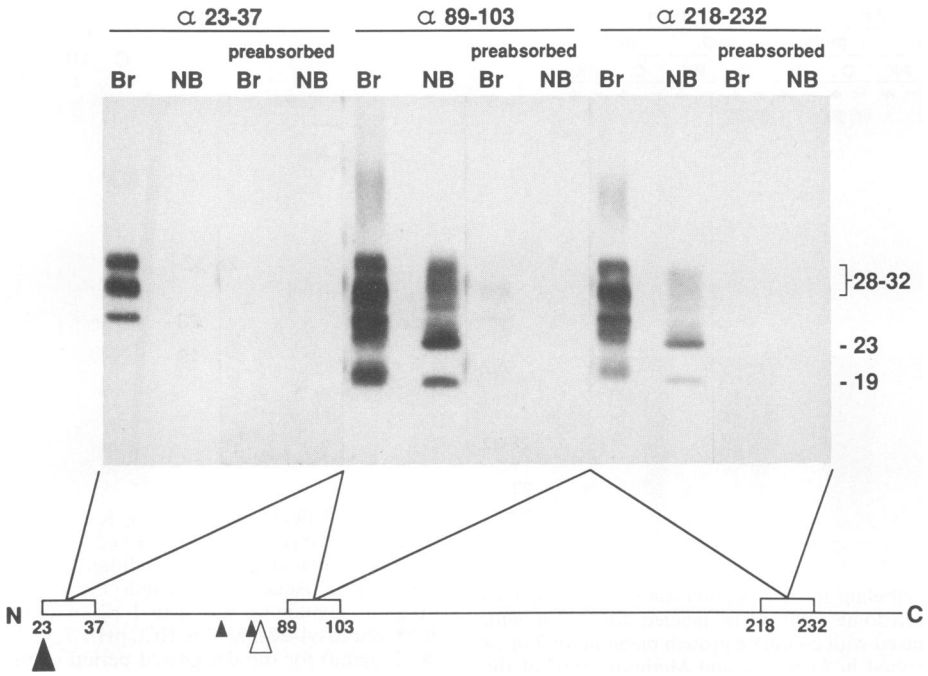

FIG. 3. Analysis of the N-terminal truncation of PrP-res by immunoblot with monospecific antisera. Aliquots of the same partially purified neuroblastoma (NB) cell PrP-res used in Fig. 2 (without proteinase K treatment) were immunoblotted using antisera (α) raised against synthetic peptides corresponding to the designated amino acid residues of the PrP sequence. As a positive control for the activity of the antisera, samples of a similar preparation of sc^+ mouse brain tissue-derived PrP-res (Br) were included. The brain tissue-derived PrP-res had been partially digested with proteinase K so that both full-length and N-terminally truncated forms of PrP-res were observed. The relative positions of the peptide antigens in the full PrP amino acid sequence (without the N-terminal signal peptide corresponding to residues 1 to 22) are diagrammed. For comparison, the major (large triangles) and minor (small triangles) N termini determined for untreated (filled triangles) and proteinase K digested (open triangle) mouse brain tissue-derived PrP-res are also shown (30). The specificity of the staining was shown by preabsorbing the antisera with the corresponding synthetic PrP peptides as described in the legend to Fig. 1. The positions of the major neuroblastoma cell PrP-res bands are designated on the right in kilodaltons. The truncated 28- to 32-kDa PrP-res of the neuroblastoma cells appears almost as large as the upper full-length brain PrP-res band identified by the α 23-37. This can be attributed to the more extensive glycosylation of PrP in neuroblastoma cells.

Inhibition of PrP-res truncation. To address the question of whether PrP-res was truncated by lysosomal or endosomal proteases and to simultaneously obtain positive evidence that PrP-res was being truncated in the intact cells rather than in the detergent lysates, we compared the SDS-PAGE mobilities of PrP-res labeled in the presence or absence of leupeptin and NH_4Cl. Leupeptin directly inhibits certain lysosomal and endosomal proteases, and NH_4Cl inhibits lysosomal proteolytic activities by raising the internal pH of these organelles (27, 48). Both of these agents inhibit the degradation of plasma membrane proteins that occurs via the endocytic pathway to the lysosomes (27, 48).

Figure 5 shows that larger PrP-res species were observed in cells treated with either 10 mM NH_4Cl or 200 μg of leupeptin per ml. More effective inhibition of truncation was achieved by using both agents simultaneously. The inhibition of PrP-res truncation by NH_4Cl provided evidence that lysosomal proteases were involved in the truncation of PrP-res. The leupeptin effect suggested the involvement of lysosomal and/or endosomal proteases. The PrP-res species detected in the cells treated with the combined inhibitors were approximately 3 kDa higher in apparent molecular mass than the corresponding proteinase K-treated species or the untreated species from the control cells. However, they remained approximately 3 to 4 kDa smaller than the corresponding PrP-sen species (Fig. 5), suggesting that the inhibition of proteolysis by these compounds was not complete and that the elongated PrP-res species still lacked about 25 to 30 amino acid residues that were present in the PrP-sen species. Furthermore, the detection of the PrP-res species of intermediate sizes with the inhibitor treatments indicates that the truncation process involves more than a single proteolytic step.

The treatments with NH_4Cl and leupeptin had no apparent effect on the cells when visualized by light microscopy. The percentage of viable cells, i.e., those capable of excluding trypan blue, was slightly decreased by the inhibitor treatments but remained greater than 90% in all cases (data not shown).

Since the inhibitors were effective when added to the living cells and not, in the case of leupeptin, when present only in the lysing buffer, the inhibitor-sensitive proteolysis occurred in the intact cells rather than in the lysate. This conclusion was also supported by the observed inhibition with NH_4Cl, which requires that the lysosomes be intact.

DISCUSSION

In the normal cycle of PrP metabolism, PrP-sen has a half-life of 3 to 6 h (8, 14, 16), which is consistent with the chase-dependent decrease in PrP-sen labeling observed in Fig. 1. Since it appears that only a small proportion of PrP-sen is released from the cell spontaneously, it is likely that most of the PrP-sen is catabolized within the cell (14). PrP-res, on the other hand, shows no evidence of turnover within the cell (8, 16). However, our present observation

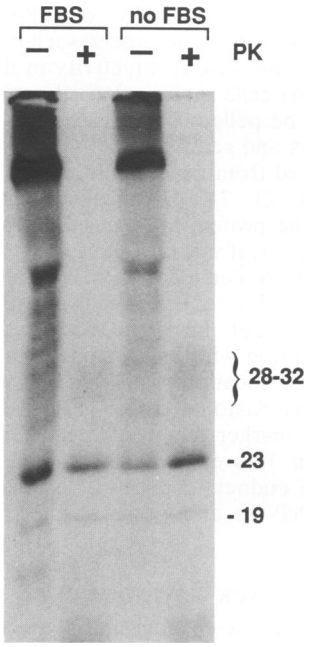

FIG. 4. PrP-res labeling in the absence of FBS. Four 25-cm^2 flasks of sc$^+$ neuroblastoma cells were labeled, chased for 24 h, and lysed as described in Materials and Methods, except that in two of the flasks the FBS was omitted from the labeling and chase media. The postnuclear supernatants from the duplicate flasks were mixed and then divided into equal parts and either treated with proteinase K (PK) or not and processed for the detection of PrP-res by immunoprecipitation as described in the legend to Fig. 1 and Materials and Methods. The apparent molecular masses of the PrP-res bands are designated on the right of the figure in kilodaltons.

that PrP-res is truncated at the N terminus indicates that it does come into contact with cellular proteases but is resistant to complete degradation.

Our inability to detect full-length PrP-res in the sc$^+$ neuroblastoma cells suggests that PrP-res is truncated at the N terminus concurrent with, or soon after, its formation. Since PrP-res is made from a cell surface precursor that is similar, if not identical, to the normal PrP-sen (16) and appears to accumulate intracellularly (8, 13, 53), it is reasonable to expect that PrP-res, or its precursor, is endocytosed. Endosomes are commonly translocated to a perinuclear location where they are fused with Golgi-derived vesicles containing proteases and acidified to form lysosomes (32). Protease-sensitive proteins contained within the lysosomes are then digested. This pathway is responsible for the normal turnover of most plasma membrane components (27) and thus is likely to account for the degradation of PrP-sen. The fact that PrP-res is truncated by leupeptin- and NH_4Cl-sensitive proteases provides evidence that PrP-res follows the endocytic pathway to the lysosomes. PrP-res may then accumulate in the lysosomes if it is not subsequently translocated to other organelles. Since PrP-res is not completely degraded in the lysosomes, its conversion to the protease-resistant state must occur prior to its exposure to proteases within endolysosomes and secondary lysosomes. Thus, it is likely that PrP-res is generated at the plasma membrane, where the protease- and phospholipase-sensitive PrP-res precursor is found, or along the endocytic pathway before exposure to proteases (16).

A question that remains is why the PrP-res species observed in the leupeptin- and NH_4Cl-treated cells did not comigrate with the PrP-sen species. The most likely explanation would be that we did not achieve complete inhibition of lysosomal and endosomal protease activity. Neither of these inhibitors completely prevents the degradation of

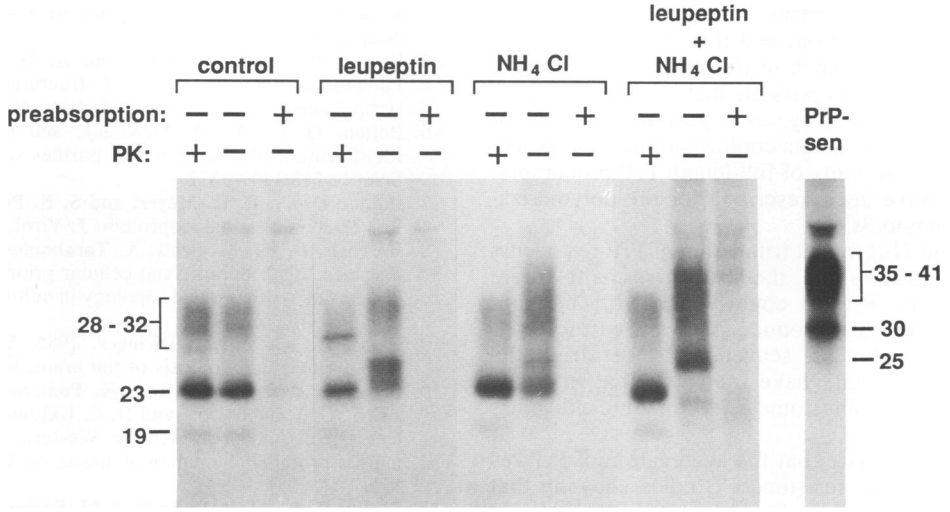

FIG. 5. Inhibition of the truncation of PrP-res. Flasks of sc$^+$ neuroblastoma cells were labeled for 2 h with Tran^{35}S-label and then chased for 18 h with complete medium. Duplicate flasks were left untreated (control) or were treated with 200 μg of leupeptin per ml, 10 mM NH_4Cl or both during the labeling and chase periods. Whereas the leupeptin was present during the entire labeling period, the NH_4Cl was added 1 h after the initiation of labeling in order to reduce its potential effects on the labeling of PrP (49). Postnuclear supernatants of the duplicate flasks were prepared and mixed. One-third was treated with proteinase K (PK; 20 μg/ml, 30 min, 37°C), and the remainder was left untreated. Both samples were then treated with 1 mM PMSF. PrP-res was then pelleted, washed with 10% Sarkosyl buffer, and immunoprecipitated with or without preabsorption of the antibody (α89-103) with the synthetic PrP peptide as described in Materials and Methods and the legend to Fig. 1. For comparison, a lane containing PrP-sen immunoprecipitated directly from a postnuclear supernatant is shown. The positions of the control PrP-res and PrP-sen species are designated in kilodaltons on the left and right sides of the panel, respectively.

plasma membrane-derived proteins (28, 31, 34). Leupeptin inhibits only a subset of the lysosomal and endocytic proteases and NH_4^+ will not affect proteases that may be active in a neutral environment within endosomes or any other organelles playing a partial role in PrP-res truncation. It remains possible that some truncation occurs in the detergent lysate, but this is unlikely because there is no evidence for the truncation of PrP-sen in the same lysates. Another possibility is that the PrP-res precursor is synthesized de novo in a partially truncated form, perhaps as a result of alternative mRNA splicing; however, the fact that full-length PrP-res is found in sc^+ brain tissue (5, 29, 30) indicates that this is not generally the case.

The combined observations that the 19- to 23- and 28- to 32-kDa PrP-res bands each lacked at least the N-terminal peptide 23-37 epitope (Fig. 5), were insensitive to further (limited) proteinase K digestion (Fig. 1B and 4), and yet contained epitopes (residues 89 to 103 and 218 to 232) bracketing most of the protease-resistent core of brain tissue-derived PrP-res, provide evidence that their diverse SDS-PAGE mobilities were not due primarily to differences in the length of their polypeptide backbone. Rather, the data support the idea that, like the multiple PrP-sen species (14), the PrP-res species differ in the amount of N-linked glycosylation they contain and that the 19-kDa PrP-res band lacks N-linked glycans altogether (16, 52). As has been noted previously (12, 52), the fact that the 19-kDa species was a component of PrP-res indicated that N-linked glycosylation was not essential for protease resistance.

Although the truncation of PrP-res observed in sc^+ neuroblastoma cells helps to define the subcellular location and kinetics of PrP-res formation, it is unclear what role the proteolysis of PrP-res may play in the biogenesis of PrP-res or in scrapie pathogenesis in vivo. Little of the brain PrP-res isolated from certain species, e.g., hamsters, is truncated beyond the signal peptidase cleavage site (5, 29). This suggests that much of the PrP-res produced in vivo is not exposed to appropriate proteases, perhaps because of impairment of lysosomal function, and that the proteolysis of PrP may have little importance in the scrapie disease process. On the other hand, it is possible that a small amount of proteolysed PrP-res could play an important role, for instance, by efficiently nucleating a conformational change and aggregation of larger amounts of full-length PrP precursors. Similar processes have been described for the polymerization of synthetic amyloids (3).

To the extent that N-terminal truncation of PrP-res occurs in scrapie-infected brain tissue, the issue arises as to where the truncation occurs. PrP-res could conceivably be truncated within the cells that produce it, in an extracellular space, or in other cells that scavenge PrP-res from the diseased tissue. Our results have indicated that in tissue culture at least, sc^+ neuroblastoma cells can quite efficiently truncate their own PrP-res.

It is tempting to speculate that the aggregation of PrP-res accounts for its protease resistance. Studies showing that PrP-res from sc^+ brain tissue is aggregated in detergent extracts (6, 23, 43) raised the question of whether the tendency to aggregate is an intrinsic property of PrP-res or a secondary effect of long-term degenerative brain pathology. For instance, PrP-res might aggregate because of pathology-induced processing or the binding of PrP-res with other pathology-associated molecules. The observation that sc^+ neuroblastoma cell PrP-res aggregates in detergent lysates (Fig. 1) (52) is consistent with the idea that the tendency to aggregate is an intrinsic property of PrP-res, since the cell cultures show no signs of scrapie-associated cytopathology.

Like PrP-res, the scrapie infectivity in detergent extracts of neuroblastoma cells is also relatively resistant to proteinase K and can be pelleted by ultracentrifugation (39). The fact that PrP-res and scrapie infectivity share these properties when derived from tissue culture cells, as well as from brain tissue (6, 23, 24, 43), provides further suggestive evidence that the protein is physically associated with the transmissible agent, if not the agent itself.

Thus, the subcellular location of PrP-res formation may also be the site of scrapie agent replication. However, it remains possible that the replication of the agent and its apparent association with PrP-res occur as separate events. In any case, the truncation of PrP-res that we have observed within sc^+ neuroblastoma cells provides a kinetic and organelle-specific marker that helps define the metabolic fate of PrP-res. Our future studies will focus on the plasma membrane and endocytic pathway as likely sites for the formation of PrP-res and perhaps, therefore, the scrapie agent.

ACKNOWLEDGMENTS

We thank Bruce Chesebro for helpful discussions and Bob Evans and Gary Hettrick for graphics assistance.

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